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Blood metal levels and related antioxidant enzyme activities in patients with Ataxia-Telangiectasia

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Running title: metals in Ataxia-Telangiectasia

23 **ABSTRACT**

24 Transition metals are co-factors for a wide range of vital enzymes, and are directly or indirectly
25 involved in the response against reactive oxygen species (ROS), which can damage cellular
26 components. Their altered homeostasis has been studied in neurodegenerative disorders such as
27 Alzheimer's disease (AD), Parkinson's disease (PD) and Amyotrophic Lateral Sclerosis (ALS), but
28 no data are available on rarer conditions.

29 We aimed at studying the role of essential trace elements in Ataxia-Telangiectasia (A-T), a rare
30 form of paediatric autosomal recessive cerebellar ataxia with altered antioxidant response. We
31 found an increased level of copper (Cu, $p=0.0002$), and a reduced level of zinc (Zn, $p=0.0002$) in
32 the blood of patients (n. 16) compared to controls, using inductively coupled plasma mass
33 spectrometry (ICP-MS). Other trace elements involved in the oxidative stress response, such as
34 manganese (Mn) and selenium (Se) were unaltered. Cu/Zn-dependent superoxide dismutase
35 (SOD1) was shown to have a 30% reduction in gene expression and 40% reduction in enzyme
36 activity upon analysis of lymphoblastoid cell lines of patients (Student's t-test, $p=0.0075$). We also
37 found a 30% reduction of Mn-SOD (SOD2; Student's t-test, $p=0.02$), probably due to a feedback
38 regulatory loop between the two enzymes. The expression of antioxidant enzymes, such as
39 erythrocyte glutathione peroxidase (*GPX1*), and *SOD2* was unaltered, whereas catalase (*CAT*) was
40 increased in A-T cells, both at the mRNA level and in terms of enzyme activity (~25%). Enhanced
41 *CAT* expression can be attributed to the high ROS status, which induces *CAT* transcription.

42 These results suggest that alterations in essential trace elements and their related enzymes may play
43 a role in the pathogenesis of A-T, although we cannot conclude if altered homeostasis is a direct
44 effect of A-T mutated genes (*ATM*). Altered homeostasis of trace elements may be more prevalent
45 in neurodegenerative diseases than previously thought, and it may represent both a biomarker and a
46 generic therapeutic target for different disorders with the common theme of altered antioxidant
47 enzyme responses associated with an unbalance of metals.

48

49 INTRODUCTION

50 Ataxia-Telangiectasia (A-T) is an autosomal recessive multisystem disorder with a frequency of
51 1/40,000–1/100,000 (Swift et al., 1986). Main clinical features include progressive cerebellar
52 degeneration leading to severe neuromotor dysfunction, oculocutaneous telangiectasia, profound
53 immunodeficiency of both humoral and cellular compartments, gonadal dysgenesis, growth
54 retardation in some patients, predisposition to malignancies (primarily lymphoreticular), high levels
55 of serum alpha-fetoprotein, and acute radiosensitivity. A-T cells show chromosomal instability,
56 premature senescence, accelerated telomere shortening, sensitivity to the cytotoxic and clastogenic
57 effects of ionizing radiation and radiomimetic chemicals, and defective activation of cell-cycle
58 checkpoints by these agents (Boder and Sedgwick, 1970; Gatti, 2001; Shiloh, 2006). The A-T
59 mutated gene (*ATM*) encodes for a serine/threonine protein kinase recruited and activated by DNA
60 double-strand breaks. ATM phosphorylates several key proteins that initiate the activation of DNA
61 damage checkpoints, leading to cell cycle arrest, DNA repair or apoptosis. Although the
62 neurodegenerative phenotype has been attributed to a defective response to DNA breaks in pre- and
63 post-mitotic neurons (Lee et al., 2001), oxidative stress and reduced anti-oxidant defence may also
64 play a role (Biton et al., 2006).

65 Correlation of the A-T phenotype with oxidative stress has been determined by molecular, *in vitro*
66 and animal studies. A-T patients show persistent oxidative stress at the cellular level, and *ATM*
67 knock-out mice exhibit increased signatures of reactive oxygen species (ROS) (Chen et al., 2003;
68 Kamsler et al., 2001; Liu et al., 2005; Watters, 2003). At the molecular level, persistent oxidative
69 stress in A-T cells was recently associated with the activation of ATM in response to oxidants (Guo
70 et al., 2010), a mechanism clearly distinct from activation by DNA breaks. Therefore, ATM plays a
71 role in redox-sensing and signalling, and the loss of redox balance in A-T may be central to the
72 neuro-pathological phenotype. Several reports have demonstrated the protective effects of low

73 molecular weight antioxidants on the A-T phenotype (Browne et al., 2004; Reliene et al., 2008;
74 Reliene and Schiestl, 2007).

75 Multiple antioxidant defence systems are present in the human body to escape the damage caused
76 by reactive oxygen species (ROS). Superoxide dismutase (SOD) acts by degrading the superoxide
77 anion ($O_2^{\cdot-}$), while catalase (CAT) and glutathione peroxidase (GPx) detoxify hydrogen peroxide
78 (H_2O_2). Trace elements are required in small concentrations as essential cofactors for the
79 antioxidant enzymes. GPx, cytoplasmic Zn/Cu-SOD (SOD1), mitochondrial Mn-SOD (SOD2) and
80 CAT enzymes contain Se, Zn/Cu, Mn and Fe as cofactors, respectively.

81 Trace metals therefore play important roles in a wide variety of biological processes, and their
82 altered homeostasis has been implicated in the aetiology of several neurodegenerative disorders
83 (Bush, 2003; Jellinger, 2013). Metals interfere with cell signalling pathways and affect growth
84 receptors, tyrosine and serine/threonine kinases, as well as nuclear transcription factors, by reactive
85 oxygen species (ROS)-dependent and ROS-independent mechanisms. In neurodegenerative
86 disorders, it is now recognized that the main underlying cause is increased oxidative stress,
87 substantiated by the findings that the protein side-chains are modified either directly by ROS or
88 reactive nitrogen species (RNS), or indirectly, by the products of lipid peroxidation. Hydroxyl
89 radical (OH^{\cdot}) is the primary ROS implicated in neurodegenerative stress, and although peroxynitrite
90 appears to be capable of hydroxyl-like activities, hydroxyl radicals mostly reflect the Fenton
91 reaction between reduced transition metals, usually iron or copper, and hydrogen peroxide (H_2O_2)
92 (Taddeo et al., 2003).

93 In Alzheimer's disease, the increased level of oxidative stress in the brain is reflected by elevated
94 levels of iron (Fe) and copper (Cu) in the brain, both of which are capable of stimulating free
95 radical formation via the Fenton reaction (Jomova et al., 2010).

96 Breakdown of metal-ion homeostasis can lead to metals binding to protein sites which are not
97 intended for metal-binding, replacement of other metals from their natural binding sites (Nelson,

1999), or to uncontrolled metal-mediated formation of deleterious free radicals (Gutteridge, 1995; Valko et al., 2007). In particular, essential trace elements such as copper, zinc and manganese, play a major role in metabolic pathways, and they have been studied in many diseases, including autoimmune, neurological and psychiatric disorders. Currently, only scarce information is available, on *in vivo* redox abnormalities in A-T patients (Aksoy et al., 2004; Reichenbach et al., 2002; Reichenbach et al., 1999). Here, we have studied the blood concentration of trace elements in A-T patients. We further explored the expression of *CAT*, *GPXI*, *SOD1* and *SOD2*, and the activity of CAT, SOD1 and SOD2 enzymes in A-T lymphoblastoid cell lines, to verify if changes in the metal concentration correlate with antioxidant enzyme activity, which is probably secondary to the alterations in their cofactor concentrations.

109

110 **MATERIALS AND METHODS**

111 *Patients*

112 We enrolled 16 A-T patients (9 males and 7 females; median age 10.6 years, range 3-23 years) diagnosed with A-T according to the diagnostic criteria of the European Society for Immunodeficiencies (ESID), and subsequently confirmed by genetic testing. None of the patients had acute infections at the time of sample collection. The control group consisted of 18 healthy individuals (10 males and 8 females, median age 13.2, age range 3-23 years old). The study was carried out in accordance with the ethical standards specified in the 1964 Declaration of Helsinki, and was approved by the internal review board of the Department of Medical Sciences (DSM-ChBU). Informed consent was obtained from patients or their legal representative.

120

121 *Blood sampling and analysis of metals*

122 Venous blood was collected in heparinized vacutainer BD tubes (Becton Dickinson Labware,
123 Franklin Lakes, USA), and stored at -20°C until required for analysis. A sub-aliquot of 1 mL of
124 blood was transferred into a 15 mL plastic tube (Falcon, Becton, Franklin Lakes, USA), then added
125 to 2mL of super-pure concentrated HNO₃ (Romil Ltd., Cambridge, UK) and microwave (MW)
126 digested in an ETHOS-Mega II oven (FKV, Bergamo, Italy), following the temperature ramp
127 program: 45 min to reach 100°C and 4 h at 100°C. The system was equipped with an optical sensor
128 to regulate the temperature. In each digestion, a blank reagent and a blood certified reference
129 material was also processed (Seronorm trace elements whole blood level 2, Sero AS, Billingstätt,
130 Norway).

131

132 *Instruments*

133 A Thermo X series II ICP-MS instrument (Thermo Scientific, Germany), with interface Ni sampler
134 and skimmer cone, was equipped with a CETAC ASX 500 Model 520 (CETAC Technologies,
135 USA) auto sampler and a peristaltic pump nebulizer.

136 Operating conditions were as follows: forward power 1.40 kW, coolant gas flow rate 13.0 L/min,
137 auxiliary gas flow rate 0.70 L/min, nebulizer gas flow rate 0.90 L/min, dwell time 10 ms, with five
138 replicates. Torch position, ion lenses and gas output were optimized daily with a tuning solution.

139 The Collision Cell Technique (CCT), performed with a Helium/Hydrogen mixture (95/5) at a flow
140 rate of 3.5 ml/min, was used to remove interferences.

141 An ETHOS 1 microwave digestion system (Milestone S.r.l, Italy) was used for acid digestion of
142 cereals and reference materials. The recoveries are shown in Table S1. The Limit Of Quantification
143 (LOQ) is the lowest concentration of the analyte that can be correctly quantified in the sample. The
144 percentage of recovery (range 80-120) is determined by dividing the value observed in the
145 analytical procedure by the reference value of the Reference Material (Table S1).

146

147

148

Table S1. Quantification limit values ($\mu\text{g/L}$), reference material values and percentages of recovery.

Element	LOQ	Seronorm trace elements whole blood level 2	Percentage of recovery
As	10	14.3 \pm 2.9	107
Be	5	5.68 \pm 0.23	108
Cd	5	5.8 \pm 0.2	105
Co	5	5.8 \pm 1.2	90
Cr	10	11.8 \pm 2.4	108
Cu	10	1,330 \pm 270	109
Fe	100	331,000 \pm 17,000	106
Mn	10	29.9 \pm 6.0	103
Ni	20	17.9 \pm 3.6	n.a.
Pb	10	310 \pm 62	102
Sb	10	30.5 \pm 6.1	92
Se	10	112 \pm 23	98
Sn	10	5.7 \pm 1.2	n.a.
Tl	10	10.3 \pm 0.3	90
Zn	100	6,500 \pm 300	90

Note: n.a: not applicable because <LOQ

149

150 *Cell culture*

151 Five A-T lymphoblastoid cell lines (LCLs) were obtained from blood samples of patients by
 152 Epstein-Barr virus (EBV) infection (Table S2). Six gender-matched control LCLs were obtained
 153 from the Human Genetics Foundation of Torino (HuGeF). LCLs were grown at 37°C and 5% CO₂
 154 in RPMI-1640 medium supplemented with 10% heat-inactivated foetal bovine serum, 2 mM L-
 155 glutamine, 0.1 mg/ml streptomycin and 100 U/ml penicillin.

Table S2. *ATM* mutations at protein level in tested cell lines.

Gender	Protein change
M	p.[(Arg2506fs)];[(Arg2506fs)]
F	p.[(Trp2109*)];[(Trp412*)]

F p.[(Ser1037fs;Lys2643_Lys2671del)];[(p.Trp1814)]
 F p.[(Met2938fs)];[*3057Glyext*28)]
 M p.[(Met1_Pro938dup)];[(Arg62_Arg111del)]

156

157 *Gene expression*

158 Total RNA was extracted using Direct-zol according to the manufacturer's protocol (Zymo
 159 Research Corporation, Irvine, USA); one milligram was retro-transcribed using M-MLV Reverse
 160 Transcriptase (Life Technologies Europe, Monza, Italy). Quantitative real-time RT-PCR to evaluate
 161 *CAT*, *GPX1*, *SOD1* and *SOD2* expression was carried out on an ABI-Prism7500 Fast instrument
 162 (Life Technologies, Europe) using the TaqMan® Universal PCR Master Mix, Universal Probe
 163 Library (UPL) technology (Roche Diagnostics, Mannheim, Germany) (Table S3).

Table S3. Primers and probes used for gene expression analysis by real-time qPCR

Assay	Primer F	Primer R	UPL probe
<i>CAT</i>	5'-gctcattttgaccgagagaga	5'-tgacctcaaagtagccaaagg	#68
<i>GPX1</i>	5'-caaccagtttgggcatcag	5'-tctcgaagagcatgaagttgg	#77
<i>SOD1</i>	5'-tcatcaatttcgagcagaagg	5'-gcaggccttcagtcagtcc	#60
<i>SOD2</i>	5'-ctggacaaacctcagcccta	5'-tgatggcttcagcaactc	#22

164

165 Experimental Ct values were normalized to the human *GUSB* gene (beta glucuronidase) or *TBP*
 166 (TATA-binding protein) Endogenous Controls (VIC®/TAMRA Probe, Life Technologies Europe).
 167 Gene expression was calculated in each sample relative to the mean of controls, using the delta-
 168 delta Ct method as described (Livak and Schmittgen, 2001). Each sample was examined in
 169 triplicate.

170

171 *Analysis of SOD isoforms activities*

172 A total of 1×10^7 cells, obtained from five A-T cell lines and four control cells, were collected and
 173 homogenized in lysis buffer (20 mM Hepes pH 7.2, 1 mM EGTA, 210 mM mannitol and 70 mM

174 sucrose). Cells were then centrifuged at 1,500 g for 5 min at 4°C. To separate the two enzymes
175 (cytosolic and mitochondrial), supernatants were centrifuged at 10,000 g for 15 min at 4°C; the
176 supernatant contained the cytosolic SOD, whereas the pellet was washed and resuspended in ice
177 cold lysis buffer to measure mitochondrial SOD. To evaluate SOD activities in the two lysates, we
178 used the Superoxide Dismutase Assay kit (Cayman, MI, USA, #706002). Analysis was performed
179 by reading the absorbance at 440-460 nm on the microplate Reader, Model 680 (Bio-
180 Rad Laboratories S.r.l., Segrate, Italy). To obtain SOD activity quantification, we compared
181 absorbance values to a Standard curve with the range 0-0.25 U/mL of the SOD standard (assayed in
182 each experiment in triplicate). Each sample was assayed in duplicate and in at least three
183 independent experiments.

184

185 *Analysis of CAT activity*

186 The day of the experiment, a total of 5×10^6 cells, obtained from three A-T cell lines and three
187 control cells, were collected by centrifugation, washed twice in PBS and homogenized in ice cold
188 lysis buffer (50 mM potassium phosphate, 1 mM EDTA, pH 7.0). Cells were centrifuged at 10,000
189 g for 15 minutes at 4°C. Supernatant was stored on ice for the assay. To evaluate CAT activities, we
190 used the Catalase Assay kit (Cayman, MI, USA, #707002) following manufacture's protocol.
191 Analysis was performed by monitoring the absorbance at 540 nm on a xMark microplate Reader
192 (Bio-Rad Laboratories S.r.l., Segrate, Italy). CAT activity was defined as the amount of CAT
193 enzyme able to produce 1.0 nmol of formaldehyde per minute at 25°C, interpolating the values in a
194 standard curve of formaldehyde ranging 0-75 μ M. Each sample was assayed in duplicate and in at
195 least three independent experiments.

196

197 *Statistical analysis*

For analysing metal concentrations, we performed the D'Agostino-Pearson normality test to determine the distribution of the values. Mean values of variables with normal distribution were reported, and comparison between control group and the patient group was conducted using the Student's t-test. If the distribution of data was not normal, variables were presented as median values (Cr, Fe, Mn, Cu, Se and Zn) and differences between the two groups were studied using the Mann-Whitney *U* test. The median concentration of each quantifiable element is shown with its standard deviation. Results were considered statistically significant at *p* values of < 0.01. Significance of gene expression and enzyme activity data was calculated using the Student's t-test (unpaired). Statistical calculations were performed using Graph Pad Statistics Software Version 6.0 (GraphPad Software, Inc., USA).

RESULTS

We measured whole blood concentration of 15 metals in A-T patients and healthy controls by using ICP-MS (Table 1).

Table 1. Median concentration of metals ($\mu\text{g/L} \pm \text{S.D}$) in the blood of A-T patients and controls.

<i>Metals</i>	<i>Patients (N=16)</i>	<i>Controls (N=18)</i>	<i>P</i>
Cr	17.3 (± 2.0)	20.7 (± 3.5)	0.3201
Cu	1,460 (± 353)	935 (± 260)	0.0002
Fe	431,350 ($\pm 1,020$)	425,271 ($\pm 2,015$)	0.5011
Mn	34.2 (± 2.1)	39.0 (± 1.4)	0.4787
Se	110 (± 50.8)	130 (± 39.9)	0.2140
Zn	4,370 (± 435)	5,760 (± 577)	0.0002

Note: In bold statistically significant metal ions.

As, Be, Cd, Co, Ni, Pb, Sb, Sn and Tl levels were below the limit of quantitation of the method. Copper levels were significantly higher in A-T patients ($p = 0.0002$) and zinc levels were significantly lower ($p = 0.0002$). As copper and zinc are co-factors for ROS detoxifying enzymes,

we measured the activity of cytosolic (Cu/Zn-isoform, SOD1), mitochondrial superoxide dismutase (Mn-isoform, SOD2) and catalase (CAT) by ELISA assay in A-T LCLs. We showed a 40% reduction of Cu/Zn-SOD isoform activity (A-T median dose: 0.66 ± 0.06 U/ml; $n = 5$ vs. CTRLs median dose: 1.0 ± 0.09 U/ml; $n = 5$; Student's t-test, $p = 0.0075$) and a 30% reduction of Mn-SOD (A-T median dose: 0.67 ± 0.08 U/ml; $n = 5$ vs. CTRLs median dose: 1.1 ± 0.13 U/ml; $n = 5$; Student's t-test, $p = 0.02$). CAT activity was increased by ~25% in patients' cells compared to controls (A-T mean dose: 1.24 ± 0.08 ; vs. CTRLs mean dose: 1.0 ± 0.8 ; Student's t-test, $p = 0.039$) (Figure 1A).

Considering the feedback regulatory loop of detoxifying enzymes, we measured the expression of the same genes and *GPXI* involved in the ROS response. Using real-time quantitative PCR, we showed a decreased SOD1 mRNA level in A-T cells vs. CTRLs (A-T median dose: 0.7 ± 0.03 ; $n = 4$. CTRLs median dose: 1.0 ± 0.05 ; $n = 4$) (Student's t-test, $p = 0.0001$). *GPXI* and *SOD2* showed levels similar to healthy controls (*GPXI*: A-T median dose: 0.95 ± 0.04 ; $n = 5$ vs. CTRLs median dose: 1.1 ± 0.08 ; $n = 5$; Student's t-test, $p = 0.02$, data not shown. *SOD2*: A-T median dose: 1.1 ± 0.06 ; $n = 5$ vs. CTRLs median dose: 1.0 ± 0.03 ; $n = 5$; Student's t-test, $p = 0.10$). Catalase mRNA level was increased in A-T cells vs. CTRLs (A-T median dose: 1.5 ± 0.09 ; $n = 5$ CTRLs median dose: 1.0 ± 0.07 ; $n=5$) (Student's t-test, $p = 0.0012$) (Figure 1B).

DISCUSSION

The current knowledge in the field of neurodegenerative diseases indicates that metal-induced and metal-enhanced formation of free radicals and other reactive species can be regarded as a common factor in determining toxicity induced by metals (Jomova et al., 2010). Many reports link the origin of Alzheimer's disease (AD), and to a lesser extent Parkinson's disease (PD), to increased oxidative stress of the brain. A role for metals in these diseases and other disorders, such as Huntington's

241 disease, amyotrophic lateral sclerosis, and prion diseases such as Creutzfeldt-Jakob disease has been
242 proposed (Bush and Curtain, 2008; Jomova et al., 2010).

243 We focused our attention on a rare form of paediatric ataxia, Ataxia-Telangiectasia, because of our
244 interest in the genetics and molecular pathogenesis of this disease (Cavalieri et al., 2008; Cavalieri
245 et al., 2006; Cavalieri et al., 2012). Among the pleiotropic features of A-T, neurodegeneration and
246 premature aging are strongly associated with accumulation of oxidative damage which may
247 contribute to degenerative processes observed in this disease (Reichenbach et al., 2002). *In vitro*, A-
248 T cells are under a constant state of oxidative stress with high ROS levels, and have an abnormal
249 response to agents inducing oxidative stress (Watters, 2003). Several groups have documented the
250 presence of high levels of oxidative damage in A-T patients, confirming previous observations
251 made in *Atm*^{-/-} mice which displayed increased levels of oxidative stress and damage (Hoche et al.,
252 2012; Kamsler et al., 2001; Schubert et al., 2004; Stern et al., 2002). In fact, brains or astrocytes
253 from *Atm*-deficient mice present high ROS levels and an increased activation of the ERK1/2 redox-
254 sensitive kinases (Liu et al., 2005). (Reliene and Schiestl, 2007) showed that the antioxidant N-
255 acetylcysteine suppresses ERK signalling and protects Purkinje cells from oxidative stress-induced
256 degeneration in *Atm*-deficient mice. Furthermore, (Stern et al., 2002) found a significantly impaired
257 level of nicotinamide adenine dinucleotide phosphate, a cofactor of antioxidant enzymes, in
258 cerebellar neurons of *Atm*^{-/-} mice. Developing neurons are rapidly proliferating and potentially able
259 to accumulate high levels of oxidants; therefore the above data provide extensive evidence that at
260 least a part of the neurological phenotype in A-T may result from ROS-deficient homeostasis
261 (Hoche et al., 2012).

262 We found increased copper and reduced zinc levels in the blood of A-T patients. Alterations in
263 copper levels may reflect many physiological and pathological conditions, including dietary factors,
264 hepatic disease, and acute and chronic infections, or it may be suggested they are associated directly
265 with ATM impairment.

266 Copper is toxic when present in excessive amounts as it can directly induce ROS production,
267 through Fenton and Haber-Weiss reactions (Halliwell, 2006). Therefore, free Cu levels must be
268 precisely regulated in the cell in order to minimize damage. The excess of Cu reported in A-T
269 patients may promote free radical-mediated pathways that, in turn, give rise to an antioxidant
270 response. In our patients, an increase in mRNA and activity of catalase may represent the first
271 process to escape an excess of ROS. However, this response seems to be insufficient.

272 Indeed, maintenance of appropriate copper levels in neurons is critical for their correct development
273 and/or function; specifically, release of copper into the synaptic cleft regulates the excitability of
274 neurons and also helps protect the neurons from excitotoxicity (Marmolino and Manto, 2010). Cu
275 dyshomeostasis has been related to neurodegenerative disorders such as Alzheimer's, and
276 amyotrophic lateral sclerosis (ALS), and it is directly involved in Mendelian disorders such as
277 Wilson and Menkes diseases (Ahuja et al., 2014).

278 Cu is also an essential component of Complex IV of the mitochondrial respiratory chain and part of
279 the ROS scavenging cell repertory, being a co-factor of the superoxide dismutase isoform present in
280 the cytosol (Cu/Zn-SOD or SOD1). SOD1 is the predominant superoxide dismutase in most cells
281 and tissues, accounting for 70–80% of the total cellular SOD activity. Its primary function is to act
282 as an antioxidant enzyme, lowering the steady-state concentration of superoxide. Over 100 different
283 mutations have been identified in the *SOD1* gene in patients diagnosed with the familial form of
284 AML (Valentine et al., 2005).

285 An equilibrated molar ratio between Cu and Zn is essential for correct function of SOD1. In the
286 presence of a Cu/Zn unbalance, equimolar Cu/Zn-SOD rapidly forms heterodimers with Zn-
287 deficient SOD leading to SOD1 deficiency. The stabilization of Zn-deficient SOD with Cu/Zn-SOD
288 has been suggested to contribute to the dominant inheritance of ALS mutations (Roberts et al.,
289 2007). We suggest that the impaired molar ratio of Cu/Zn seen in A-T patients may be the basis of
290 the SOD1 functional reduction in A-T cells.

291 Eukaryotic systems have evolved defence mechanisms against free radicals and the manganese
292 superoxide dismutase (Mn-SOD or SOD2) is a key mitochondrial antioxidant enzyme, coded by the
293 *SOD2* gene, which catalyses the conversion of superoxide anions to hydrogen peroxide (Flynn and
294 Melov, 2013). Loss of SOD2 activity can result in numerous pathological phenotypes in
295 metabolically-active tissues, particularly within the central nervous system. SOD2 is potentially
296 involved in the progression of neurodegenerative diseases, such as stroke and Alzheimer's and
297 Parkinson's diseases, as well as its potential role in "normal" age-related cognitive decline (Clausen
298 et al., 2010). In this study, we found that blood manganese concentrations were comparable in
299 patients and controls, although SOD2 enzyme activity assays showed a 30% decrease in
300 comparison to the control group.

301 Conversely, reduction of zinc may lead to several deleterious effects. The decrease of Zn
302 concentrations in the blood could be a result of Zn accumulation in tissues, along with dietary
303 factors. Zinc is an essential metal implicated in the functioning of more than 200 enzymes; it plays
304 an important role in axonal and synaptic transmission and is necessary for nucleic acid metabolism
305 and brain tubulin growth and phosphorylation. In physiological concentrations, zinc exhibits
306 neuroprotective activity, although an unbalance of zinc homeostasis has been reported in a number
307 of brain processes, which can then lead to the onset of chronic pathologies such as depression,
308 schizophrenia, AD, PD, aging, or ALS (Szewczyk, 2013). High concentrations of zinc are
309 neurotoxic (Choi et al., 1988; Cote et al., 2005; Perry et al., 1997; Plum et al., 2010), and its
310 deficiency has been reported in the plasma of AD patients, and it is hypothesized that a deficiency
311 of Zn could be one of the contributing factors in the development of AD (Constantinidis, 1991;
312 Religa et al., 2006).

313 It is interesting to note that many A-T patients display a primary immunodeficiency, and zinc
314 deficiency may play a role in modulating this phenotype (Lynn and Wong, 1997; Prasad et al.,
315 2007). Zinc is essential for the maintenance of immune function and for the development and

316 function of neutrophils, macrophages, and natural killer cells. Thus, zinc deficiency leads to the
317 reduction of thymulin, interleukin-2, and interferon-gamma, and the increase in production of pro-
318 inflammatory cytokines: zinc deficiency is also associated with a higher incidence of infections.
319 A hypothesis for the protective role of antioxidants considers the induction of synthesis of
320 metallothioneins (MTs). These proteins contain a large number of thiol groups, which are effective
321 in the reduction of ROS formation (Valko et al., 2005). MTs are zinc-binding proteins involved in
322 the regulation of the transport, storage and transfer of zinc to various enzymes and transcription
323 factors (DiGirolamo et al., 2010; Liuzzi and Cousins, 2004).
324 Finally, similarly to our findings, zinc was found to be significantly reduced in the plasma of
325 children with Type 1 Diabetes Mellitus (T1DM) (Salmonowicz et al., 2014), the activity of SOD1
326 was significantly reduced and CAT activity was significantly increased. (Shukla et al., 2006)
327 proposed that in diseases associated with chronic oxidative stress, ROS impair the Cu/Zn-SOD
328 function by reducing intracellular Cu ions found in protein compounds such as metallothionein and
329 SOD.

330

331 **CONCLUSIONS**

332 Mutations that inactivate wide-ranging regulators such as *ATM*, the gene mutated in A-T, are
333 expected to affect many cellular systems and cause serious disruption of cellular homeostasis. The
334 clinical and cellular phenotypes of such disorders also indicate ongoing deleterious processes,
335 marked by slowly progressing degeneration of specific tissues and occasionally by signs of
336 premature aging. A possible contributor to these processes, which are also seen in various
337 neurodegenerative diseases and aging tissues, is oxidative stress, reflected by elevated levels of
338 ROS. A-T cells show poor cellular anti-oxidant defences and increased oxidant sensitivity
339 compared to normal cells, and *ATM* partly functions as an oxidative stress sensor. Accumulating
340 evidence suggests that oxidative stress is involved in the pathogenesis of A-T.

341 In line with these observations, we detected alterations in the essential trace elements copper and
342 zinc, which are involved in the oxidative-stress response, in the blood of A-T patients, which was
343 associated with transcriptional and functional alterations of ROS-detoxifying enzymes (CAT, SOD1
344 and SOD2) in patients' cell lines.

345 Our results suggest that zinc and copper homeostasis may play a role in the pathology of A-T, as
346 summarized in figure 2. The initial determinant of the dyshomeostasis of these metals is unknown,
347 and may be *ATM* deficiency itself. In turn, they contribute to an altered antioxidant defence system,
348 impairing SOD1 and SOD2 activity. Enhanced catalase expression is a marker of increased ROS
349 activity. Indeed Zn depletion, due to its antioxidant properties, may act to further increase oxidative
350 damage.

351 Cu/Zn alterations may therefore be suggested as biomarkers of A-T, and open to future works on
352 other ataxias of genetic origin. Furthermore, given the unbalance and the antioxidant properties of
353 Zn, dietary supplementation with this metal, which has also been proposed in other studies (Bao et
354 al., 2008; Prasad et al., 2007), would be an interesting therapeutic possibility that should be
355 explored in future experiments.

356

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363

364 **FIGURE LEGENDS**

365 **Figure 1.** Enzymatic activity and expression analysis of ROS-detoxifying enzymes in A-T
366 lymphoblasts. A. In the upper panels, enzymatic activity of the related proteins was measured by
367 ELISA assay and expressed as relative activity vs. controls (Superoxide Dismutase Assay kit,
368 Cayman, MI, USA, and Catalase Assay kit, Cayman, MI, USA, #707002). CAT showed a 20%
369 increase in activity in A-T patients. Both SOD1 and SOD2 enzymes showed a reduced activity of
370 30-40% in A-T cell lines (* = $p < 0.05$; ** = $p < 0.01$). B. In the lower panels, expression of *CAT*,
371 *SOD1* and *SOD2* genes was tested by real-time PCR. Reference genes for normalization were
372 *GUSB* for *CAT*, and *SOD2* and *TBP* for *SOD1*. Fold-changes were significant for *CAT* and *SOD1*
373 genes and concordant with activity (* = $p < 0.05$). CTRLs: controls; A-T: Ataxia-Telangiectasia
374 patients.

375

376 **Figure 2.** Schematic summary of the role of Cu and Zn metals in A-T cells. A Cu/Zn unbalance,
377 which may be directly associated with *ATM* mutations, or secondary to unknown causes, can affect
378 A-T cell survival by increasing ROS directly via Cu oxidative damage, and by SOD1/SOD2
379 impairment. ROS increase induces a higher CAT transcription in the attempt to reduce ROS stress.

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382 REFERENCES

- 383 Ahuja, A., et al., 2014. Copper mediated neurological disorder: Visions into amyotrophic lateral
384 sclerosis, Alzheimer and Menkes disease. *J Trace Elem Med Biol*.
- 385 Aksoy, Y., et al., 2004. Antioxidant enzymes in red blood cells and lymphocytes of ataxia-
386 telangiectasia patients. *Turk J Pediatr*. 46, 204-7.
- 387 Bao, B., et al., 2008. Zinc supplementation decreases oxidative stress, incidence of infection, and
388 generation of inflammatory cytokines in sickle cell disease patients. *Transl Res*. 152, 67-80.
- 389 Biton, S., et al., 2006. Nuclear ataxia-telangiectasia mutated (ATM) mediates the cellular response
390 to DNA double strand breaks in human neuron-like cells. *J Biol Chem*. 281, 17482-91.
- 391 Boder, E., Sedgwick, R. P., 1970. Ataxia-telangiectasia. (Clinical and immunological aspects).
392 *Psychiatr Neurol Med Psychol Beih*. 13-14, 8-16.
- 393 Browne, S. E., et al., 2004. Treatment with a catalytic antioxidant corrects the neurobehavioral
394 defect in ataxia-telangiectasia mice. *Free Radic Biol Med*. 36, 938-42.
- 395 Bush, A. I., 2003. The metallobiology of Alzheimer's disease. *Trends Neurosci*. 26, 207-14.
- 396 Bush, A. I., Curtain, C. C., 2008. Twenty years of metallo-neurobiology: where to now? *Eur*
397 *Biophys J*. 37, 241-5.
- 398 Cavalieri, S., et al., 2008. Large genomic mutations within the ATM gene detected by MLPA,
399 including a duplication of 41 kb from exon 4 to 20. *Ann Hum Genet*. 72, 10-8.
- 400 Cavalieri, S., et al., 2006. ATM mutations in Italian families with ataxia telangiectasia include two
401 distinct large genomic deletions. *Hum Mutat*. 27, 1061.
- 402 Cavalieri, S., et al., 2012. Deep-intronic ATM mutation detected by genomic resequencing and
403 corrected in vitro by antisense morpholino oligonucleotide (AMO). *Eur J Hum Genet*.
- 404 Chen, S., et al., 2003. ATM's leucine-rich domain and adjacent sequences are essential for ATM to
405 regulate the DNA damage response. *Oncogene*. 22, 6332-9.
- 406 Choi, D. W., et al., 1988. Zinc neurotoxicity in cortical cell culture. *Neuroscience*. 24, 67-79.
- 407 Clausen, A., et al., 2010. Prevention of cognitive deficits and brain oxidative stress with superoxide
408 dismutase/catalase mimetics in aged mice. *Neurobiol Aging*. 31, 425-33.
- 409 Constantinidis, J., 1991. The hypothesis of zinc deficiency in the pathogenesis of neurofibrillary
410 tangles. *Med Hypotheses*. 35, 319-23.
- 411 Cote, A., et al., 2005. Cell type-specific action of seizure-induced intracellular zinc accumulation in
412 the rat hippocampus. *J Physiol*. 566, 821-37.
- 413 DiGirolamo, A. M., et al., 2010. Randomized trial of the effect of zinc supplementation on the
414 mental health of school-age children in Guatemala. *Am J Clin Nutr*. 92, 1241-50.

415 Flynn, J. M., Melov, S., 2013. SOD2 in mitochondrial dysfunction and neurodegeneration. *Free*
 416 *Radic Biol Med.* 62, 4-12.
 417 Gatti, R. A., 2001. The inherited basis of human radiosensitivity. *Acta Oncol.* 40, 702-11.
 418 Guo, Z., et al., 2010. ATM activation in the presence of oxidative stress. *Cell Cycle.* 9, 4805-11.
 419 Gutteridge, J. M., 1995. Lipid peroxidation and antioxidants as biomarkers of tissue damage. *Clin*
 420 *Chem.* 41, 1819-28.
 421 Halliwell, B., 2006. Oxidative stress and neurodegeneration: where are we now? *J Neurochem.* 97,
 422 1634-58.
 423 Hoche, F., et al., 2012. Neurodegeneration in ataxia telangiectasia: what is new? What is evident?
 424 *Neuropediatrics.* 43, 119-29.
 425 Jellinger, K. A., 2013. The relevance of metals in the pathophysiology of neurodegeneration,
 426 pathological considerations. *Int Rev Neurobiol.* 110, 1-47.
 427 Jomova, K., et al., 2010. Metals, oxidative stress and neurodegenerative disorders. *Mol Cell*
 428 *Biochem.* 345, 91-104.
 429 Kamsler, A., et al., 2001. Increased oxidative stress in ataxia telangiectasia evidenced by alterations
 430 in redox state of brains from Atm-deficient mice. *Cancer Res.* 61, 1849-54.
 431 Lee, Y., et al., 2001. Ataxia telangiectasia mutated-dependent apoptosis after genotoxic stress in the
 432 developing nervous system is determined by cellular differentiation status. *J Neurosci.* 21,
 433 6687-93.
 434 Liu, N., et al., 2005. ATM deficiency induces oxidative stress and endoplasmic reticulum stress in
 435 astrocytes. *Lab Invest.* 85, 1471-80.
 436 Liuzzi, J. P., Cousins, R. J., 2004. Mammalian zinc transporters. *Annu Rev Nutr.* 24, 151-72.
 437 Livak, K. J., Schmittgen, T. D., 2001. Analysis of relative gene expression data using real-time
 438 quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods.* 25, 402-8.
 439 Lynn, W. S., Wong, P. K., 1997. Possible control of cell death pathways in ataxia telangiectasia. A
 440 case report. *Neuroimmunomodulation.* 4, 277-84.
 441 Marmolino, D., Manto, M., 2010. Pregabalin antagonizes copper-induced toxicity in the brain: in
 442 vitro and in vivo studies. *Neurosignals.* 18, 210-22.
 443 Nelson, N., 1999. Metal ion transporters and homeostasis. *EMBO J.* 18, 4361-71.
 444 Perry, D. K., et al., 1997. Zinc is a potent inhibitor of the apoptotic protease, caspase-3. A novel
 445 target for zinc in the inhibition of apoptosis. *J Biol Chem.* 272, 18530-3.
 446 Plum, L. M., et al., 2010. The essential toxin: impact of zinc on human health. *Int J Environ Res*
 447 *Public Health.* 7, 1342-65.

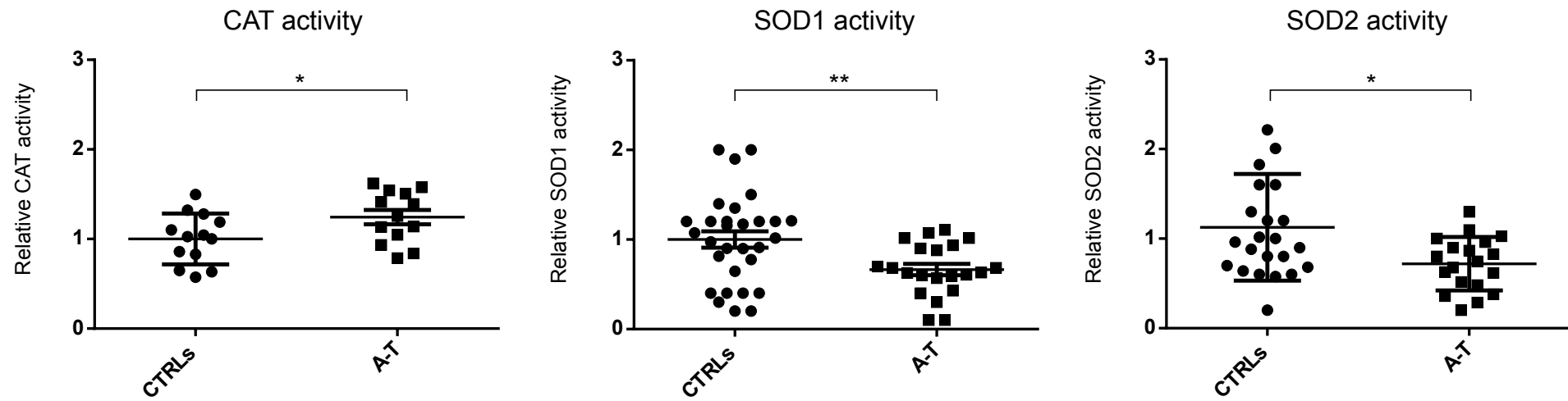
448 Prasad, A. S., et al., 2007. Zinc supplementation decreases incidence of infections in the elderly:
 449 effect of zinc on generation of cytokines and oxidative stress. *Am J Clin Nutr.* 85, 837-44.
 450 Reichenbach, J., et al., 2002. Elevated oxidative stress in patients with ataxia telangiectasia.
 451 *Antioxid Redox Signal.* 4, 465-9.
 452 Reichenbach, J., et al., 1999. Anti-oxidative capacity in patients with ataxia telangiectasia. *Clin Exp*
 453 *Immunol.* 117, 535-9.
 454 Reliene, R., et al., 2008. Effects of antioxidants on cancer prevention and neuromotor performance
 455 in Atm deficient mice. *Food Chem Toxicol.* 46, 1371-7.
 456 Reliene, R., Schiestl, R. H., 2007. Antioxidants suppress lymphoma and increase longevity in Atm-
 457 deficient mice. *J Nutr.* 137, 229S-232S.
 458 Religa, D., et al., 2006. Elevated cortical zinc in Alzheimer disease. *Neurology.* 67, 69-75.
 459 Roberts, B. R., et al., 2007. Structural characterization of zinc-deficient human superoxide
 460 dismutase and implications for ALS. *J Mol Biol.* 373, 877-90.
 461 Salmonowicz, B., et al., 2014. Trace elements, magnesium, and the efficacy of antioxidant systems
 462 in children with type 1 diabetes mellitus and in their siblings. *Adv Clin Exp Med.* 23, 259-
 463 68.
 464 Schubert, R., et al., 2004. Cancer chemoprevention by the antioxidant tempol in Atm-deficient
 465 mice. *Hum Mol Genet.* 13, 1793-802.
 466 Shiloh, Y., 2006. The ATM-mediated DNA-damage response: taking shape. *Trends Biochem Sci.*
 467 31, 402-10.
 468 Shukla, N., et al., 2006. Does oxidative stress change ceruloplasmin from a protective to a
 469 vasculopathic factor? *Atherosclerosis.* 187, 238-50.
 470 Stern, N., et al., 2002. Accumulation of DNA damage and reduced levels of nicotine adenine
 471 dinucleotide in the brains of Atm-deficient mice. *J Biol Chem.* 277, 602-8.
 472 Swift, M., et al., 1986. The incidence and gene frequency of ataxia-telangiectasia in the United
 473 States. *Am J Hum Genet.* 39, 573-83.
 474 Szewczyk, B., 2013. Zinc homeostasis and neurodegenerative disorders. *Front Aging Neurosci.* 5,
 475 33.
 476 Taddeo, M. A., et al., Metal-Catalyzed Redox Activity in Neurodegenerative Disease. In: P. Zatta,
 477 (Ed.), *Metal Ions and Neurodegenerative Disorders* World Scientific, 2003, pp. 1-14.
 478 Valentine, J. S., et al., 2005. Copper-zinc superoxide dismutase and amyotrophic lateral sclerosis.
 479 *Annu Rev Biochem.* 74, 563-93.

480 Valko, M., et al., 2007. Free radicals and antioxidants in normal physiological functions and human
481 disease. *Int J Biochem Cell Biol.* 39, 44-84.
482 Valko, M., et al., 2005. Metals, toxicity and oxidative stress. *Curr Med Chem.* 12, 1161-208.
483 Watters, D. J., 2003. Oxidative stress in ataxia telangiectasia. *Redox Rep.* 8, 23-9.
484

Figure 1.

A

Enzymes activity (ELISA assay)



B

Gene expression (real-time PCR assay)

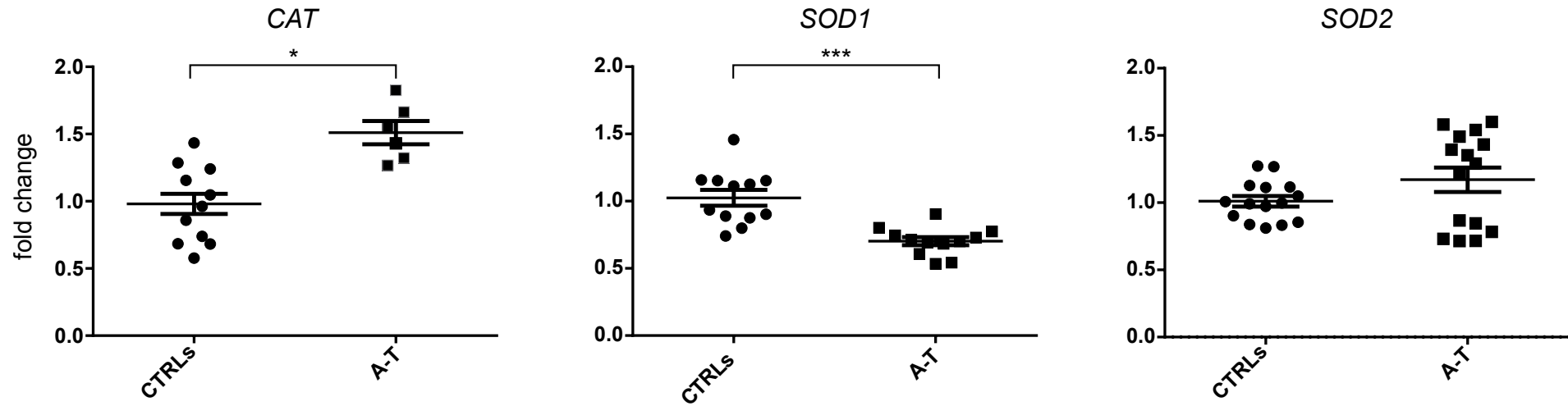


Figure 2

